NOVEL SURFACE EXPOSED PROTEINS FROM CHLAMYDIA PNEUMONIAE

The present invention relates to the identification of
members of a gene family from the human respiratory pathogen
Chlamydia pneumoniae, encoding surface exposed membrane
5   proteins of a size of approximately 89-101 kDa and of 56-57
kDa, preferably about 89.6-100.3 kDa and about 56.1 kDa. The
invention relates to the novel DNA sequences, the deduced
amino acid sequences of the corresponding proteins and the
use of the DNA sequences and the proteins in diagnosis of
10   infections caused by *C. pneumoniae*, in pathology, in
epidemiology, and as vaccine components.

GENERAL BACKGROUND

C. pneumoniae is an obligate intracellular bacteria
(Christiansen and Birkelund (1992); Grayston et al. (1986)).
15   It has a cell wall structure as Gram negative bacteria with
an outer membrane, a periplasmic space, and a cytoplasmic
membrane. It is possible to purify the outer membrane from
Gram negative bacteria with the detergent sarkosyl. This
fraction is named the 'outer membrane complex (OMC)' (Caldwell
20   et al. (1981)). The COMC (Chlamydia outer membrane complex)
of *C. pneumoniae* contains four groups of proteins: A high
molecular weight protein 98 kDa as determined by SDS-PAGE, a
double band of the cysteine rich outer membrane protein 2
(Omp2) protein of 62/60 kDa, the major outer membrane protein
25   (MOMP) of 38 kDa, and the low-molecular weight lipo-protein
Omp3 of 12 kDa. The Omp2/Omp3 and MOMP proteins are present
in COMC from all Chlamydia species, and these genes have been
cloned from both *C. trachomatis*, *C. psittaci* and *C.
pneumoniae*. However, the gene encoding 98 kDa protein from *C.
30   pneumoniae* COMC have not been characterized or cloned.

The current state of *C. pneumoniae* serology and detection

C. pneumoniae is an obligate intra-cellular bacteria
belonging to the genus Chlamydia which can be divided into

four species: *C. trachomatis, C. pneumoniae, C. psittaci* and *C.pecorum*. Common for the four species is their obligate intra cellular growth, and that they have a biphasic life cycle, with an extracellular infectious particle (the

5 elementary body, EB), and an intercellular replicating form (the reticulate body, RB). In addition the Chlamydia species are characterized by a common lipopolysaccharide (LPS) epitope that is highly immunogenic in human infection. *C. trachomatis* is causing the human ocular infection (trachoma)

10 and genital infections. *C. psittaci* is a variable group of animal pathogens where the avian strains can occasionally infect humans and give rise to a severe pneumonia (ornithosis). The first *C. pneumoniae* isolate was obtained from an eye infection, but it was classified as a non-typable

15 Chlamydia. Under an epidemic outbreak of pneumonia in Finland it was realized that the patients had a positive reaction in the Chlamydia genus specific test, (the lygranum test), and the patients showed a titre increase to the untyped Chlamydia isolates. Similar isolates were obtained in an outbreak of

20 upper respiratory tract infections in Seattle, and the Chlamydia isolates were classified as a new species, *Chlamydia pneumoniae* (Grayston et al. (1989)). In addition, *C. pneumoniae* is suggested to be involved in the development of atherosclerotic lesions and for initiating bronchial

25 asthma (Kuo et al. (1995)). These two conditions are thought to be caused by either chronic infections, by a hypersensitivity reaction, or both.

Diagnosis of *Chlamydia pneumoniae* infections

Diagnosis of acute respiratory tract infection with *C.*

30 *pneumoniae* is difficult. Cultivation of *C. pneumoniae* from patient samples is insensitive, even when proper tissue culture cells are selected for the isolation. A *C. pneumoniae* specific polymerase chain reaction (PCR) has been developed by Campbell et al.(1992).

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Even though *Chlamydia pneumoniae* has in several studies been detected by this PCR it is debated whether this method is suitable for detection under all clinical situations. The reason for this is, that the cells carrying *Chlamydia*
5   *pneumoniae* in acute respiratory infections have not been determined, and that a chronic carrier state is expected but it is unknown in which organs and cells they are present. Furthermore, the PCR test is difficult to perform due to the low yield of these bacteria and due to the presence of
10   inhibitory substances in the patient samples. Therefore, it will be of great value to develop sensitive and specific sero-diagnostics for detecting both acute and chronic infections. Sero-diagnosis of Chlamydia infections is currently based on either genus specific tests as the
15   Lygranum test and ELISA, measuring the antibodies to LPS, or the more species specific tests where antibodies to purified EBs are measured by microimmuno fluorescence (Micro-IF) (Wang et al. (1970)). However, the micro-IF method is read by microscopy, and in order to ensure correct readings the
20   result must be compared to the results with *C. trachomatis* used as antigen due to the cross-reacting antibodies to the common LPS epitope. Thus, there exists in the art an urgent need for development of reliable methods for species specific diagnosis of *Chlamydia pneumoniae*, as has been expressed in
25   Kuo et al. (1995); "..a rapid reliable laboratory test of infection for the clinical laboratory is a major need in the field". Furthermore, the possible involvement of *C. pneumoniae* in atherosclerosis and bronchial asthma clearly warrants the development of an effective vaccine.

30   DETAILED DISCLOSURE OF THE INVENTION

The present invention aims at providing means for efficient diagnosis of infections with *Chlamydia pneumoniae* as well as the development of effective vaccines against infection with this microorganism. The invention thus relates to species
35   specific diagnostic tests for infection in a mammal, such as a human, with *Chlamydia pneumoniae*, said tests being based on

the detection of antibodies against surface exposed membrane proteins of a size of approximately 89-101 kDa and of 56-57 kDa, preferably of about 89.6-100.3 kDa and about 56.1 kDa (the range in size of the deduced amino acid sequences was from 100.3 to 89.6 except for Omp13 with the size of 56.1 kDa), or the detection of nucleic acid fragments encoding such proteins or variants or subsequences thereof. The invention further relates to the amino acid sequences of proteins according to the invention, to variants and subsequences thereof, and to nucleic acid fragments encoding these proteins or variants or subsequences thereof. The present invention further relates to antibodies against proteins according to the invention. The invention also relates to the use of nucleic acid fragments and proteins according to the invention in diagnosis of *Chlamydia pneumoniae* and vaccines against *Chlamydia pneumoniae*.

Prior to the disclosure of the present invention only a very limited number of genes from *C. pneumoniae* had been sequenced. These were primarily the genes encoding known *C. trachomatis* homologues: MOMP, Omp2, Omp3, Kdo-transferase, the heat shock protein genes GroEl/Es and DnaK, a ribonuclease P homologue and a gene encoding a 76 kDa protein of unknown function. The reason why so few genes have been cloned to date is the very low yield of *C. pneumoniae* which can be obtained after purification from the host cells. After such purification the DNA must be purified from the EBs, and at this step the *C. pneumoniae* DNA can easily be contaminated with host cell DNA. In addition to these inherent difficulties, it is exceedingly difficult to cultivate *C. pneumoniae* and use DNA technology to produce expression libraries with very low amounts (few μg) of DNA. It has been known since 1993 (Melgosa et al., 1993) that a 98 kDa protein is present in OMC from *C. pneumoniae*. Even though the protein bands of 98 kDa was mentioned to be part of the OMC of *C. pneumoniae* by Melgosa, the gene sequences and thus the deduced amino acid sequences have not been determined. Only

bands originating from Chlamydia pneumoniae proteins in general separated by SDS-PAGE are describe therein.

However, the gene encoding this protein has not been determined before the present invention. Only a very weak or no reaction with patient sera can be observed to the 98 kDa

5 protein (Campbell et al. 1990) and prior to the work of the present inventors it has not been recognized that the 89-101 kDa proteins are surface exposed or that they in fact is immunogenic (see below). In this report it is described that a number of human serum samples reacts with a C. pneumoniae protein that in SDS-PAGE migrate as 98 kDa. The protein was not further characterized and it is therefore not in conflict with the present

10 application.

Campbell et al. (1990) described that sera from four patients from which Chlamydia pneumonia was isolated reacted with bands of 98 kDa in immunoblotting using whole-cell lysates. They also showed that no proteins with similar molecular weights were recognised

15 by serum samples in either Chlamydia trachomatis or Chlamydia psittaci and they therefore suggest that the protein present in the 98 kDa band could be used as a potential diagnostic tool for the recognition of Chlamydia pneumoniae infection. The protein content within the 98 kDa region was not further characterised and its localisation within the Chlamydia was not shown.

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Halme et al. (1997) described the presence of human T-cell epitopes in C. pneumoniae proteins of 92-98 kDa. The proteins were eluted from SDS-PAGE of total chlamydia proteins but the identity of the proteins were not determined.

25 Use of antibodies to screen expression libraries is a well known method to clone fragments of genes encoding antigenic parts of proteins. However, since patient sera do not show a significant reaction with the 98 kDa protein it has not been possible to use patient serum to clone the proteins.

30 It was known that monoclonal antibodies generated by the inventors reacted with conformational epitopes on the surface of C. pneumoniae and that they also reacted with C. pneumoniae OMC by immuno-electron microscopy (Christiansen et al. 1994). Furthermore, the 98 kDa protein is the only unknown protein from the C. pneumoniae OMC (Melgosa et al. 1993). The present inventors chose to take an unconventional step in order

AMENDED SHEET

to clone the gene encoding the hitherto unknown 98 kDa protein: C. pneumoniae OMC was purified and the highly immunogenic conformational epitopes were destroyed by SDS-treatment of the antigen before immunization. Thereby an antibody (PAB 150) to less immunogenic linear epitopes was obtained. This provided the possibility to obtain an

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antiserum which could detect the protein, and it was shown that a gene family encoding the 89-101 kDa and 56 proteins according to the invention could be detected in colony blotting of recombinant *E. coli*.

5   Mice infected with *C. pneumoniae* generate antibodies to the proteins identified by the inventors and named Omp4-15, but do not recognize the SDS treated heat denatured antigens normally used for SDS-PAGE and immunoblotting. However, a strong reaction was seen if the antigen was not heat
10 denatured. It is therefore highly likely that if a similar reaction is seen in connection with human infections the antigens of the present invention will be of invaluable use in sero-diagnostic tests and may very likely be used as a vaccine for the prevention of infections.
15

By generating antibodies against COMC from *C. pneumoniae* a polyclonal antibody (PAB 150) was obtained which reacted with all the proteins. This antibody was used to identify the genes encoding the 89.6-101.3 kDa and 56.1 kDa proteins in an
20 expression library of *C. pneumoniae* DNA. A problem in connection with the present invention was that a family comprising a number of similar genes were found in *C. pneumoniae*. Therefore, a large number of different clones were required to identify clusters of fragments. Only because
25 the rabbit antibody generated by the use of SDS-denatured antigens contained antibodies to a high number of different epitopes positioned on different members of the protein family did the inventors succeed in cloning and sequencing four of the genes. One gene was fully sequenced, a second was
30 sequenced except for the distal part and shorter fragments of two additional genes were obtained by this procedure. To obtain the DNA sequence of the additi al genes and to sea for more members of the gene family long range PCR with primers derived from the sequenced genes, and primers from
35 the genes already published in the database were used. This approach gave rise to the detection of additional eight genes belonging to this family. The genes were situated in two gene

clusters: Omp12,11,10,5,4,13 and 14 in one cluster and
Omp6,7,8,9 and 15 in the second. Full sequence was obtained
from Omp4,5,6,7,8,9,10,11 and 13, and partial sequence of
Omp12,14. Omp13 was a truncated gene of 1545 nucleotides. The
5   rest of the full length genes were from 2526 (Omp7) to 2838
(Omp15) nucleotides. The deduced amino acid sequences
revealed putative polypeptides of 89.6 to 100.3 kDa, except
for Omp13 of 56.1 kDa. Alignment of the deduced amino acid
sequences showed a maximum identity of 49% (Omp5/Omp9) when
10   all the sequences were compared. Except for Omp13, the lowest
homology was to Omp7 with no more than 34% identity to any of
the other amino acid sequences. The scores for Omp13 was from
29-32% to all the other sequences.

In the present context SEQ ID Nos. 1 and 2 correspond to
15   Omp4, SEQ ID Nos 3 and 4 correspond to Omp5, SEQ ID Nos 5 and
6 correspond to Omp6, SEQ ID Nos 7 and 8 correspond to Omp7,
SEQ ID Nos 9 and 10 correspond to Omp8, SEQ ID Nos 11 and 12
correspond to Omp9, SEQ ID Nos 13 and 14 corresponds to
Omp10, SEQ ID Nos 15 and 16 corresponds to Omp11, SEQ ID Nos
20   17 and 18 corresponds to Omp12, SEQ ID Nos 19 and 20
corresponds to Omp13, SEQ ID Nos 21 and 22 corresponds to
Omp14, and SEQ ID Nos 23 and 24 corresponds to Omp15.

The estimated size of the Omp proteins of the of the present
invention are listed in the following. Omp 4 has a size of
25   98.9 kDa, Omp5 has an estimated size of 97.2 kDa, Omp6 has an
estimated size of 100.3 kDa, Omp7 has an estimated size of
89.7 kDa, Omp8 has an estimated size of 90.0 kDa, Omp9 has an
estimated size of 96.7 kDa, Omp10 has an estimated size of
98.4 kDa, Omp11 has an estimated size of 97.6 kDa, Omp13 has
30   an estimated size of 56.1 kDa, Omp 12 and 14 being partial.

Furthermore, SEQ ID No 25 is a subsequence of SEQ ID No 3,
SEQ ID No 26 is a subsequence of SEQ ID No 4, SEQ ID No 27 is
a subsequence of SEQ ID No 5, SEQ ID No 28 is a subsequence
of SEQ ID No 6, SEQ ID No 29 is a subsequence of SEQ ID No 7,
35   and SEQ ID No 30 is a subsequence of SEQ ID No 8.

Part of the omp proteins were expressed as fusion proteins, and mice polyclonal monospecific antibodies against the proteins were produced. The antibodies reacted with the surface of C. pneumoniae in both immunofluorescence and

5   immunoelectron microscopy. This shows for the first time that the 89-101 kDa and 56-57 kDa protein family in C. pneumoniae comprises surface exposed outer membrane proteins. This important finding leads to the realization that members of the 89-101 kDa and 56-57 kDa C. pneumoniae protein family are

10 good candidates for the development of a sero diagnostic test for C. pneumoniae, as well as the development of a vaccine against infections with C. pneumoniae based on using these proteins. Furthermore, the proteins may be used as epidemiological markers, and polyclonal monospecific sera

15 against the proteins can be used to detect C. pneumoniae in human tissue or detect C. pneumoniae isolates in tissue culture. Also, the genes encoding the 89-101 kDa and 56-57 kDa such as the 89.6-100.3 kDa and 56.1 protein family may be used for the development of a species specific diagnostic

20 test based on nucleic acid detection/amplification.

The full length Omp4 was cloned into an expression vector system that allowed expression of the Omp4 polypeptide. This polypeptide was used as antigen for immunization of a rabbit. Since the protein was purified under denaturing condition the

25 antibody did not react with the native surface of C. pneumoniae, but it reacted with a 98 kDa protein in immunoblotting where purified C. pneumoniae EB was used as antigen. Furthermore, the antibody reacted in paraffin embedded sections of lung tissue from experimentally infected

30 mice.

A broad aspect of the present invention relates to a species specific diagnostic test for infection of a mammal, such as a human, with Chlamydia pneumoniae, said test comprising detecting in a patient or preferable in a patient sample the

35 presence of antibodies against proteins from the outer membrane of Chlamydia pneumoniae, said proteins being of a

molecular weight of 89-101 kDa or 56-57 kDa, or detecting the
presence of nucleic acid fragments encoding said outer
membrane proteins or fragments thereof.

5   In the context of the present application, the term "patient
     sample" should be taken to mean an amount of serum from a
     patient, such as a human patient, or an amount of plasma from
     said patient, or an amount of mucosa from said patient, or an
     amount of tissue from said patient, or an amount of
10   expectorate, forced sputum or a bronchial aspirate, an amount
     of urine from said patient, or an amount of cerebrospinal
     fluid from said patient, or an amount of atherosclerotic
     lesion from said patient, or an amount of mucosal swaps from
     said patient, or an amount of cells from a tissue culture
15   originating from said patient, or an amount of material which
     in any way originates from said patient. The in vivo test in
     a human according to the present invention includes a skin
     test known in the art such as an intradermal test, e.g
     similar to a Mantaux test. In certain patients being very
20   sensitive to the test, such as is often the case with
     children, he test could be non-invasive, such as a
     superficial test on the skin, e.g. by use of a plaster

     In the present context, the term 89-101 kDa protein means
     proteins normally present in the outer membrane of *Chlamydia*
25   *pneumoniae*, which in SDS-PAGE can be observed as one or more
     bands with an apparent molecular weight substantially in the
     range of 89-101 kDa. From the deduced amino acid sequences
     the molecular size varies from 89.6 to 100.3 kDa.

     Within the scope of the present invention are species
30   specific sero-diagnostic tests based on the usage of the
     genes belonging to the gene family disclosed in the present
     application.

     Preferred embodiments of the present invention relate to
     species specific diagnostic tests according to the invention,
35   wherein the outer membrane proteins have sequences selected

from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ ID NO: 24.

5   When used in connection with proteins according to the present invention the term "variant" should be understood as a sequence of amino acids which shows a sequence similarity of less than 100% to one of the proteins of the invention. A variant sequence can be of the same size or it can be of a
10   different size as the sequence it is compared to. A variant will typically show a sequence similarity of preferably at least 50%, preferably at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90%, 95% or 98%.

The term "sequence similarity" in connection with sequences
15   of proteins of the invention means the percentage of identical and conservatively changed amino acid residues (with respect to both position and type) in the proteins of the invention and an aligned protein of equal of different length. The term "sequence identity" in connection with
20   sequences of proteins of the invention means the percentage of identical amino acid with respect to both position and type in the proteins of the invention and an aligned protein of equal of different length.

Within the scope of the present invention are subsequences of
25   one of the proteins of the invention, meaning a consecutive stretch of amino acid residues taken from SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 , or SEQ ID NO: 24. A subsequence will
30   typically comprise at least 100 amino acids, preferably at least 80 amino acids, more preferably at least 70 amino acids, such as 50 amino acids. It might even be as small as 10-50 amino acids, such as 20-40 amino acids, e.g. about 30 amino acids.A subsequence will typically show a sequence
35   homology of at least 50%, preferably at least 60%, more

preferably at least 70%, such as at least 80%, e.g. at least 90%, 95% or 98%.

Diagnostic tests according to the invention include immunoassays selected from the group consisting of a direct
5   or indirect EIA such as an ELISA, an immunoblot technique such as a Western blot, a radio immuno assay, and any other non-enzyme linked antibody binding assay or procedure such as a fluorescence, agglutination or precipitation reaction, and nephelometry.

10   A preferred embodiment of the present invention relates to species specific diagnostic tests according to the invention, said test comprising an ELISA, wherein antibodies against the proteins of the invention or fragments thereof are detected in samples.

15   A preferred embodiment of the invention, is an ELISA based on detection in samples of antibodies against proteins of the invention. The ELISA may use proteins of the invention, or variants thereof, i.e. the antigen, as coating agent. An ELISA will typically be developed according to standard
20   methods well known in the art, such as methods described in "Antibodies; a laboratory manual", Ed. David Lane Harlow, Cold Spring Habor laboratories (1988), which is hereby incorporated by reference.

Recombinant proteins will be produced using DNA sequences
25   obtained essentially using methods described in the examples below. Such DNA sequences, comprising the entire coding region of each gene in the gene family of the invention, will be cloned into an expression vector from which the deduced protein sequence can be purified. The purified proteins will
30   be analyzed for reactivity in ELISA using both monoclonal and polyclonal antibodies as well as sera from experimentally infected mice and human patient sera.

From the experimentally infected mice sera it is known that non-linear epitopes are recognized predominantly. Thus, it is contemplated that different forms of purification schemes known in the art will be used to analyze for the presence of

5 discontinuous epitopes, and to analyze whether the human immune response is also directed against such epitopes.

Preferred embodiments of the present invention relate to species specific diagnostic tests according to the invention, wherein the nucleic acid fragments have sequences selected

10 from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, and SEQ ID NO: 23.

In connection with nucleic acid fragments according to the

15 present invention the term "variant" should be understood as a sequence of nucleic acids which shows a sequence homology of less than 100%. A variant sequence can be of the same size or it can be of a different size as the sequence it is compared to. A variant will typically show a sequence

20 homology of at least 50%, preferably at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90%, 95% or 98%.

The term "sequence homology" in connection with nucleic acid fragments of the invention means the percentage of matching

25 nucleic acids (with respect to both position and type) in the nucleic acid fragments of the invention and an aligned nucleic acid fragment of equal or different length.

In order to obtain information concerning the general distribution of each of the genes according to the present

30 invention, PCR will be performed for each gene on all available *C. pneumoniae* isolates. This will provide information on the general variability of the genes or nucleic acid fragments of the invention. Variable regions will be sequenced. From patient samples PCR will be used to

amplify variable parts of the genes for epidemiology. Non-variable parts will be used for amplification by PCR and analyzed for possible use as a diagnostic test. It is contemplated that if variability is discovered, PCR of

5   variable regions can be used for epidemiology. PCR of non-variable regions can be used as a species specific diagnostic test. Using genes encoding proteins known to be invariable in all known isolates prepared as targets for PCR to genes encoding proteins with unknown function.

10   Particularly preferred embodiments of the present invention, relate to diagnostic tests according to the invention, wherein detection of nucleic acid fragments is obtained by using nucleic acid amplification, preferably polymerase chain reaction (PCR).

15   Within the scope of the present invention is a PCR based test directed at detecting nucleic acid fragments of the invention or variants thereof. A PCR test will typically be developed according to methods well known in the art and will typically comprise a PCR test capable of detecting and differentiating

20   between nucleic acid fragments of the invention. Preferred are quantitative competitive PCR tests or nested PCR tests. The PCR test according to the invention will typically be developed according to methods described in detail in EP B 540 588, EP A 586 112, EP A 643 140 OR EP A 669 401, which

25   are hereby incorporated by reference.

Within the scope of the present invention are variants and subsequences of one of the nucleic acid fragments of the invention, meaning a consecutive stretch of nucleic acids taken from SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID

30   NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 19, SEQ ID NO: 21, or SEQ ID NO: 23. A variant or subsequence will preferably comprise at least 100 nucleic acids, preferably at least 80 nucleic acids, more preferably at least 70 nucleic acids, such as at least 50 nucleic acids.

35   It might even be as small as 10-50 nucleic acids, such as

20-40 nucleic acids, e.g. about 30 nucleic acids. A
subsequence will typically show a sequence homology of at
least 30%, preferably at least 60%, more preferably at least
70%, such as at least 80%, e.g. at least 90%, 95% or 98%. The
5   shorter the subsequence, the higher the required homology.
Accordingly, a subsequence of 100 nucleic acids or lower must
show a homology of at least 80%.

A very important aspect of the present invention relates to
proteins of the invention derived from Chlamydia pneumoniae
10   having amino acid sequences selected from the group
consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ
ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID
NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ
ID NO: 24 having a sequence similarity of at least 50%,
15   preferably at least 60%, more preferably at least 70%, such
as at least 80%, e.g. at least 90%, 95% or 98% and a similar
biological function.

By the term "similar biological function" is meant that the
protein shows characteristics similar with the proteins
20   derivable from the membrane proteins of Chlamydia pneumoniae.
Such proteins comprise repeated motifs of GGAI (at least 2,
preferable at least 3 repeats) and/or conserved positions of
tryptophan, (w).

Comparison of the DNA sequences from genes encoding Omp4-15
25   shows that the overall similarity between the individual
genes ranges between 43-55%. Comparison of the amino acid
sequences of Omp4-15 shows 34-49% identity and 53-64%
similarity. The homology is generally scattered along the
entire length of the deduced amino acids. However, as seen
30   from figure 8 A - J there are some regions in which the
homology is more pronounced. This is seen in the repeated
sequence where the sequence GGAI is repeated 4-7 times in the
genes. It is interesting that the DNA homology is not
conserved for the sequences encoding the four amino acids
35   GGAI. This may indicate a functional role of this part of the

protein and indicates that the repeated structure did not
occur by a duplication of the gene. In addition to the four
amino acid repeats GGAI a region from amino acid 400 to 490
has a higher degree of homology than the rest of the protein,

5   with the conserved sequence FYDPI occurring in all sequences.
As further indication of similarity in function the amino
acid tryptophan (W) is perfectly conserved at 4-6
localizations in the C-terminal part of the protein.

Since none of the genes and deduced amino acid sequences of

10   the invention are identical the following is within the scope
of the present invention; production of monospecific
antibodies, the use of said antibodies for characterizing
which C. pneumoniae proteins are expressed, the use of said
antibodies for characterizing at which time during

15   developmental life cycle said C. pneumoniae proteins are
expressed, and the use of said antibodies for characterizing
the precise cellular localization of said C. pneumoniae
proteins. Also within the scope of the present invention is
the use of monospecific antibodies against proteins of the

20   invention for determining which part of said proteins is
surface exposed and how proteins in the C. pneumoniae COMC
interact with each other.

Preferred embodiments of the present invention relate to

25   polypeptides which comprise subsequences of the proteins of
the invention, said subsequences comprising the sequence
GGAI. Further preferred embodiments of the present invention
relate to polypeptides which comprise subsequences of the
proteins of the invention, said subsequences comprising the

30   sequence FSGE.

Polypeptides according to the invention will typically be of
a length of at least 6 amino acids, preferably at least 15
amino acids, preferably at least 20 amino acids, preferably
at least 25 amino acids, preferably at least 30 amino acids,

35   preferably at least 35 amino acids, preferably at least 40
amino acids, preferably at least 45 amino acids, preferably

at least 50 amino acids, preferably at least 55 amino acids, preferably at least 100 amino acids.

A very important aspect of the present invention relates to nucleic acid fragments of the invention derived from
5   *Chlamydia pneumoniae*, variants and subsequences thereof.

Another important aspect of the present invention relates to antibodies against the proteins according to the invention, such antibodies including polyclonal monospecific antibodies and monoclonal antibodies against proteins with sequences
10   selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ ID NO: 24.

A very important aspect of the present invention relates to
15   diagnostic kits for the diagnosis of infection of a mammal, such as a human, with *Chlamydia pneumoniae*, said kits comprising one or more proteins with amino acid sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO:
20   12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ ID NO: 24.

Another very important aspect of the present invention relates to diagnostic kits for the diagnosis of infection of a mammal, such as a human, with *Chlamydia pneumoniae*, said
25   kits comprising antibodies against a protein with an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ ID NO: 24.
30   Antibodies included in a diagnostic kit according to the invention can be polyclonal or monoclonal or a mixture hereof.

Still another very important aspect of the present invention
relates to diagnostic kits for the diagnosis of infection of
a mammal, such as a human, with *Chlamydia pneumoniae*, said
kits comprising one or more nucleic acid fragments with

5   sequences selected from the group consisting of SEQ ID NO: 1,
SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ
ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ
ID NO: 19, SEQ ID NO: 21, and SEQ ID NO: 23.

An aspect of the present invention relates to a composition

10 for immunizing a mammal, such as a human, against *Chlamydia
pneumoniae*, said composition comprising one or more proteins
with amino acid sequences selected from the group consisting
of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8,
SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16,

15 SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ ID NO:
24.

An important role for the proteins of the invention in
prevention of infection of a mammal, such as a human, with *C.
pneumoniae* is expected. Thus proteins of the invention,

20 including variants and subsequences will be produced,
typically by using recombinant techniques, and will then be
used as an antigen in immunization of mammals, such as
rabbits. Subsequently, the hyper immune sera obtained by the
immunization will be analyzed for protection against *C.*

25 *pneumoniae* infection using a tissue culture assay. In
addition it is contemplated that monoclonal antibodies will
be produced, typically using standard hybridoma techniques,
and analyzed for protection against infection with *C.
pneumoniae*.

30 It is envisioned that particularly interesting and
immunogenic epitopes will be found in connection with the
proteins of the invention, which will comprise subsequences
of said proteins. It is preferred to use polypeptides
comprising such subsequences of the proteins of the invention

in immunizing a mammal, such as a human, against *Chlamydia pneumoniae*.

An important aspect of the present invention relates to the
use of proteins with sequences selected from the group
5   consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ
ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID
NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ
ID NO: 24 in diagnosis of infection of a mammal, such as a
human, with *Chlamydia pneumoniae*.

10 A preferred embodiment of the present invention relates to
the use of proteins according to the invention in an
undenatured form, in diagnosis of infection of a mammal, such
as a human, with *Chlamydia pneumoniae*.

A very important aspect of the present invention relates to
15 the use of proteins with sequences selected from the group
consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ
ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID
NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, and SEQ
ID NO: 24, for immunizing a mammal, such as a human, against
20 *Chlamydia pneumoniae*.

A preferred embodiment of the present invention relates to
the use of proteins according to the invention in an
undenatured form, for immunizing a mammal, such as a human,
against *Chlamydia pneumoniae*.

25 A very important aspect of the present invention relates to
the use of nucleic acid fragments with nucleotide sequences
selected from the group consisting of SEQ ID NO: 1, SEQ ID
NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO:
11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO:
30 19, SEQ ID NO: 21, and SEQ ID NO: 23 for immunizing a mammal,
such as a human, against *Chlamydia pneumoniae*.

It is envisioned that one type of vaccine against *C. pneumoniae* will be developed by using gene-gun vaccination of mice. Typically, different genetic constructs containing nucleic acid fragments, combinations of nucleic acid

5   fragments according to the invention will be used in the gene-gun approach. The mice will then subsequently be analyzed for production of both humoral and cellular immune response and for protection against infection with *C. pneumoniae* after challenge herewith.

10   In line with this, the invention also relates to the uses of the proteins of the invention as a pharmaceutical (a vaccine) as well as to the uses thereof for the preparation of a vaccine against infections with *Chlamydia pneumoniae*.

Preparation of vaccines which contain protein sequences as
15   active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspen-
20   sions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredi-
25   ent. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which
30   enhance the effectiveness of the vaccines.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some
35   cases, oral formulations. These compositions take the form of

solutions, suspensions, tablets, pills, capsules, sustained
release formulations or powders and contain 10-95% of active
ingredient, preferably 25-70%, and optionally a suitable
carrier.

5   The protein sequences may be formulated into the vaccine as
neutral or salt forms known in the art. The vaccines are
administered in a manner compatible with the dosage
formulation, and in such amount as will be therapeutically
effective and immunogenic. The quantity to be administered
10   depends on the subject to be treated. Suitable dosage ranges
are of the order of several hundred micrograms active
ingredient per vaccination with a preferred range from about
0.1 μg to 1000 μg. The immune response may be enhanced if the
vaccine further comprises an adjuvant substance as known in
15   the art. Other possibilities involve the use of
immunomodulating substances such as lymphokines (e.g. IFN-γ,
IL-2 and IL-12) or synthetic IFN-γ inducers such as poly I:C
in combination with the above-mentioned adjuvants.

It is also possible to produce a living vaccine by introdu-
20   cing, into a non-pathogenic microorganism, at least one
nucleic acid fragment encoding a protein fragment or protein
of the invention, and effecting expression of the protein
fragment or the protein on the surface of the microorganism
(e.g. in the form of a fusion protein including a membrane
25   anchoring part or in the form of a slightly modified protein
or protein fragment carrying a lipidation signal which allows
anchoring in the membrane). The skilled person will know how
to adapt relevant expression systems for this purpose.

Another part of the invention is based on the fact that
30   recent research have revealed that a DNA fragment cloned in a
vector which is non-replicative in eukaryotic cells may be
introduced into an animal (including a human being) by e.g.
intramuscular injection or percutaneous administration (the
so-called "gene gun" approach). The DNA is taken up by e.g.
35   muscle cells and the gene of interest is expressed by a

promoter which is functioning in eukaryotes, e.g. a viral
promoter, and the gene product thereafter stimulates the
immune system. These newly discovered methods are reviewed in
Ulmer et al., 1993, which hereby is included by reference.

5   Thus, a nucleic acid fragment encoding a protein or protein
    of the invention may be used for effecting *in vivo* expression
    of antigens, *i.e.* the nucleic acid fragments may be used in
    so-called DNA vaccines. Hence, the invention also relates to
    a vaccine comprising a nucleic acid fragment encoding a
10 protein fragment or a protein of the invention, the vaccine
    effecting *in vivo* expression of antigen by an mammal, such as
    a human, to whom the vaccine has been administered, the
    amount of expressed antigen being effective to confer
    substantially increased resistance to infections with
15 *Chlamydia pneumoniae* in an mammal, such as a human.

    The efficacy of such a "DNA vaccine" can possibly be enhanced
    by administering the gene encoding the expression product
    together with a DNA fragment encoding a protein which has the
    capability of modulating an immune response. For instance, a
20 gene encoding lymphokine precursors or lymphokines (e.g. IFN-
    γ, IL-2, or IL-12) could be administered together with the
    gene encoding the immunogenic protein fragment or protein,
    either by administering two separate DNA fragments or by
    administering both DNA fragments included in the same vector.
25 It is also a possibility to administer DNA fragments compri-
    sing a multitude of nucleotide sequences which each encode
    relevant epitopes of the protein fragments and proteins
    disclosed herein so as to effect a continuous sensitization
    of the immune system with a broad spectrum of these epitopes.

30 The following experimental non-limiting examples are intended
    to illustrate certain features and embodiments of the inven-
    tion.

LEGENDS TO FIGURES

Figure 1. The figure shows electron microscopy of negative stained purified *C. pneumoniae* EB (A) and purified OMC (B).

Figure 2. The figure shows silver stained 15% SDS-PAGE of
5　purified EB and OMC. Lane 1, purified *C. pneumoniae* EB; lane 2, *C. pneumoniae* OMC; lane 3, purified *C. trachomatis* EB; and lane 4 *C. trachomatis* OMC.

Figure 3. The figure shows immunoblotting of *C. pneumoniae* EB separated by 10% SDS-PAGE, transferred to nitrocellulose and
10　reacted with rabbit anti *C. pneumoniae* OMC.

Figure 4. The figure shows coomassie blue stained 7.5% SDS-PAGE of recombinant pEX that were detected by the rabbit anti *C. pneumoniae* serum. Arrow indicated the localization of the 117 kDa b-galactosidase protein.

15　Figure 5. The figure shows immunoblotting of recombinant pEX colones detected by colony blotting separated by 7.5% SDS-PAGE and transferred to nitrocellulose and reacted with rabbit anti *C. pneumoniae* OMC. Lane 1, seablue molecular weight standard. Lane 2-6 pEX clones cultivated at 42°C to
20　induce the production of the b-galactosidase fusion proteins.

Figure 6. The figure shows sequence strategy for Omp4 and Omp5. Arrows indicates primers used for sequencing.

Figure 7. *C pneumoniae* omp genes. The genes are arranged in two clusters. In cluster 1 Omp12, 11, 10, 5, 4, 13, and 14
25　are found. In cluster 2 are found Omp6, 7, 8, 9, and 15.

Figure 8 A - J. The figure shows alignment of *C. pneumoniae* Omp4-15, using the program pileup in the GCG package.

Figure 9. The figure shows immunofluorescence of *C. pneumoniae* infected HeLa, 72 hrs. after infection, reacted

with mouse monospecific anti-serum against pEX3-36 fusion
protein. pEX3-36 is a part of the Omp5 gene.

Figure 10. The figure shows immunoblotting of *C. pneumoniae*
EB, lane 1-3 heated to 100°C in SDS-sample buffer, lane 4-6
5   unheated. Lane 1 reacted with rabbit anti *C. pneumoniae* OMC;
lane 2 and 4 pre-serum; lane 3 and 5 polyclonal rabbit anti
pEX1-1 fusion protein; lane 6 MAb 26.1.

Figure 11. The figure shows immunoblotting of *C. pneumoniae*
EB, lane 1-4 heated to 100oC in SDS-sample buffer, lane 5-6
10 unheated. Reacted with serum from C57-black mice 14 days
after infection with 10^7 CFU of *C. pneumoniae*. Lane 1 and 5
mouse 1; lane 2 and 6 mouse 2; lane 3 and 5 mouse 3; and lane
4 and 8 mouse 4.

Figure 12. The figure shows immunohistochemistry analysis of
15 mouse lung tissue with *C. pneumoniae* inclusions present both
in the bronchial epithelium and in the lung parenchyma
(arrows).

EXAMPLE 1

Cloning of the genes encoding the 98/95 kDa *C. pneumoniae* COMC proteins

Purification of *C. pneumonia* EBs and COMC

5   *C. pneumoniae* was cultivated in HeLa cells. Cultivation was
    done according to the specifications of Miyashita and
    Matsumoto (1992), with the modification that centrifugation
    of supernatant and of the later precipitate and turbid bottom
    layer was carried out at 100,000 X g. The microorganism
10 attached to the HeLa cells by 30 minutes of centrifugation at
    1000 x g, after which the cells were incubated in RPMI 1640
    medium (Gibco BRL, Germany cat No. 51800-27), containing 5%
    foetal calf serum (FCS, Gibco BRL, Germany Cat No. 10106.169)
    gentamicin for two hours at 37°C in 5% CO2 atmosphere. The
15 medium was changed to medium that in addition contained 1 mg
    per ml of cycloheximide. After 48 hours of incubation a
    coverslip was removed from the cultures and the inclusion was
    tested with an antibody specific for *C. pneumoniae* (MAb 26.1)
    (Christiansen et al. 1994) and a monoclonal antibody specific
20 for the species *C. trachomatis* (MAb 32.3, Loke diagnostics,
    Århus Denmark) to ensure that no contamination with *C.
    trachomatis* had occurred. The HeLa cells were tested by
    Hoechst stain for Mycoplasma contamination as well as by
    culture in BEa and BEg medium (Freund et al., 1979). Also the
25 *C. pneumoniae* stocks were also tested for Mycoplasma
    contamination by cultivation in BEa and BEg medium. No
    contamination with *C. trachomatis*, Mycoplasmas or bacteria
    were detected in cultures or cells. 72 hours post-infection
    the monolayer was washed in PBS, the cells were loosened in
30 PBS with a rubber policeman, and the Chlamydia were liberated
    from the host cell by sonication. The *C. pneumoniae* EBs and
    RBs were purified on discontinuous density gradients
    (Miyashita et al. (1992)). The purity of the Chlamydia EBs
    were verified by negative staining and electronmicroscopy
35 (Figure 1), only particles of a size of 0.3 to 0.5 mm were

detected in agreement with the structure of *C. pneumonia* EBs.
The purified Chlamydia EBs were subjected to sarkosyl
extraction as described by Caldwell et al (1981) with the
modification that a brief sonication was used to suspend the
5   COMC. The purified COMC was tested by electronmicroscopy and
negative staining (Figure 1), where a folded outer membrane
complex was seen.

SDS-PAGE analysis of purified EBs and COMC

The proteins from purified EBs and *C. pneumoniae* OMC were
10   separated on 15% SDS-polyacrylamide gel, and the gel was
silver stained (Figure 2), in lane 1 it is seen that the
purified EBs contain major proteins of 100/95 kDa and a
protein of 38 kDa, in the purified COMC (lane 2) these two
protein groups are also dominant. In addition, proteins with
15   a molecular weight of 62/60 kDa, 55 kDa, and 12 kDa have been
enriched in the COMC preparation. When the purified *C.*
pneumoniae EBs are compared to purified *C. trachomatis* EB
(lane 3) it is seen that predominant protein in the *C.*
trachomatis EB is the major outer membrane protein (MOMP),
20   and it is also the dominant band in the COMC preparation of
C. trachomatis (lane 4), and Omp2 of 60/62 kDa as well as
Omp3 at 12 kDa are seen in the preparation. However, no major
bands with a size of 100/95 kDa are detected as in the *C.*
pneumoniae COMC preparation.

25   **Production of rabbit polyclonal antibodies against *C.***
pneumoniae COMC

To ensure production of rabbit antibodies that would
recognize all the *C. pneumoniae* proteins in immuno-blotting
and colony-blotting 10 μg of COMC antigen was dissolved in 20
30   μl of SDS sample buffer and thereafter divided into 5 vials.
The dissolved antigen was further diluted in one ml of PBS
and one ml of Freund incomplete adjuvant (Difco laboratories,
USA cat. No. 0639-60-6) and injected into the quadriceps
muscle of a New Zealand white rabbit. The rabbit was given

three times intramuscular injections at an interval of one week, and after further three weeks the dissolved COMC protein, diluted in one ml PBS was injected intravenously, and the procedure was repeated two weeks later. Eleven weeks

5 after the beginning of the immunization, the serum was obtained from the rabbit. Purified *C. pneumoniae* EBs were separated by SDS-PAGE, and the proteins were electrotransferred to nitrocellulose membrane. The membrane was blocked and immunostained with the polyclonal COMC

10 antibody (Figure 3). The serum recognized proteins with a size of 100/95, 60 and 38 kDa in the EB preparation. This is in agreement with the sizes of the outer membrane proteins.

Cloning of the COMC proteins

Due to the cultivation of *C. pneumoniae* in HeLa cells,
15 contaminating host cell DNA could be present in the EB preparations. Therefore, the purified EB preparations were treated with DNAse to remove contaminating DNA. The *C. pneumoniae* DNA was then purified by CsCl gradient centrifugation. The *C. pneumoniae* DNA was partially digested
20 with Sau3A and the fractions containing DNA fragments with a size of approx. 0.5 to 4.0 kb were cloned into the expression vector system pEX (Boehringer, Germany cat. No. 1034 766, 1034 774, 1034 782). The pEX vector system has a β-galactosidase gene with multiple cloning sites in the 3'end
25 of the β-galactosidase gene. Expression of the gene is regulated by the PR promoter, so the protein expression can be induced by elevating the temperature from 32 to 42°C. The colonies of recombinant bacteria were transferred to nitrocellulose membranes, and the temperature was increased
30 to 42°C for two hours. The bacteria were lysed by placing the nitrocellulose membranes on filters soaked in 5% SDS. The colonies expressing outer membrane proteins were detected with the polyclonal antibody raised against *C. pneumoniae* COMC. The positive clones were cultivated in suspension and
35 induced at 42°C for two hours. The protein profile of the clones were analysed by SDS-PAGE, and increases in the size

of the induced b-galactosidase were observed (Figure 4). In
addition, the proteins were electrotransferred to
nitrocellulose membranes, and the reaction with the
polyclonal serum against COMC was confirmed (Figure 5).

5   Sequencing of positive COMC clones

To characterize the pEX clones, the inserted C. pneumoniae
DNA was sequenced. The resulting DNA sequences were searched
against the prokaryotic sequences in the GenEmbl database.
The search identified 6 clones as part of the Omp2 gene, and
10 2 clones as part of the Omp3 gene, and 2 clones as part of
the MOMP gene, indicating that COMC proteins had been
successfully cloned. Furthermore, 32 clones were obtained,
containing DNA sequences not found in the GenEmbl database.
These sequences could, however, be clustered in two contics
15 of 6 and 4 clones, and three clones were identical. In
addition 19 clones were found with no overlap to the contics
(Figure 7). To obtain more sequence data for the genes, C.
pneumoniae DNA was totally digested with BamHI restriction
enzyme, and the fragments were cloned into the vector
20 pBluescript. The ligated DNA was electrotransformed into E.
coli XL1-Blue and selected on plates containing Ampicillin.
The recombinant bacterial colonies were transferred to a
nitrocellulose membrane, and colony hybridisation was
performed using the inserts of pEX 1-1 clone as a probe. A
25 clone containing a single BamHI fragment of 4.5 kb was found,
and the hybridisation to the probe was confirmed by Southern
blotting. The insert of the clone was sequenced
bi-directionally using synthetic primers for approx. each 300
bp. The sequence of the BamHI fragment made it possible to
30 join the two contics of pEX clones. Totally, together with
the pEX clones it was possible to assemble 6.5 kb DNA
sequence, encoding two new COMC proteins. (Figure 6)

Additional sequences were obtained by PCR performed on
purified C. pneumoniae DNA with primers both from the known
35 Omp genes and from other known genes. The obtained PCR

products were sequenced, The sequence organisation is shown
in Fig. 7. Additional 8 Omp genes were detected. The
alignment of the deduced amino acid sequences are shown in
Fig. 8 A and B.

5   Analysis of DNA sequence

The DNA sequence encoding the Omp4-15 proteins with a size of
89.6-100.3 kDa (and for Omp13: 56.1 kDa). Omp4 and Omp5 were
transcribed in opposite directions. Downstream Omp4 a
possible termination structure was located. The 3'end of the
10   Omp5 gene was not cloned due to the presence of the BamHI
restriction enzyme site positioned within the gene. The
translated DNA sequence of Omp4 and Omp5 was compared by use
of the gap programme in the GCG package (Wisconsin package,
version 8.1-UNIX, August 1995, sequence analysis software
15   package). The two genes had an amino acid identity of 41%
(similarity 61%), and a possible cleavage site for signal
peptidase 1 was present at amino acid 17 in Omp4 and amino
acid 25 in Omp5. When the amino acid sequence encoded by two
other pEX clones were compared to the sequence of Omp4 and
20   Omp5 they also had amino acid homology to the genes. It is
seen that the two clones have homology to the same area in
the Omp4 and Omp5 proteins. Consequently, the pEX clones must
have originated from two additional genes. Therefore these
genes were named Omp6 and Omp7. Similar analyses were
25   performed with the other genes. In contrast to what was seen
for Omp4 and 5 none of the other putative omp proteins had a
cleavage site for signal peptides.

EXAMPLE 2

Polyclonal monospecific antibodies against pEX fusion
30   proteins and full length recombination + Omp4

To investigate the topology of the Omp4-7 proteins,
representative pEX clones, were selected from each gene. The
fusion proteins of β-galactosidase/omp were induced, and the

proteins were partially purified as inclusion bodies. Balb/c
mice were immunized three times intramuscular with the
antigens at an interval of one week, and after six weeks the
serum was obtained from the mice. HeLa cells were infected

5   with the *C. pneumoniae*. 72 hours after the infection the
mono-layers were fixed with 3.7% formaldehyde. This treatment
makes the outer membrane of the Chlamydia impermeable for
antibodies due to the extensive cross-linking of the outer
membrane proteins by the formaldehyde. The HeLa cells were

10 permeabilized with 0.2% Triton X100, the monolayers were
washed in PBS, then incubated with 20% (v/v) FCS to
inactivate free radicals of the formaldehyde. The mice sera
were diluted 1:100 PBS with 20% (v/v) FCS and incubated with
the monolayers for half an hour. The monolayers were washed

15 in PBS and secondary FITCH conjugated rabbit anti mouse serum
was added for half an hour, and the monolayers were washed
and mounted. Several of the antibodies reacted strongly with
the EBs in the inclusions (Figure 9). In spite of the
formaldehyde fixation it could not be excluded that the

20 surface of the EB was changed by the treatments, so that the
antibodies could get access to the Omp4-7. Therefore, the
reaction was confirmed by immuno-electron microscopy with the
antibody raised against clone pEX3-36. Purified EB of *C.
pneumoniae* were absorbed to carbon coated nickel grids. After

25 the absorption the grids were washed with PBS and blocked in
0.5% Ovalbumin dissolved in PBS. The antibodies were diluted
1:100 in the same buffer and incubated for 30 minutes. The
grids were washed in PBS. Rabbit anti mouse Ig conjugated
with 10nm colloidal gold diluted in PBS containing 1% gelatin

30 was added to the grids for half an hour. The grids were
washed in 3 x PBS with 1% gelatin and 3 times in PBS, the
grids were contrastained with 0.7% phospho tungstic acid. The
grids were analysed in a Jeol 1010 electron microscope at 40
kV. It was seen that the gold particles were covering the

35 surface of the purified EB. Because the *C. pneumoniae* EBs
were not exposed to any detergent or fixation under either
the purification or the reaction with antibodies, these

results show that the cloned proteins have surface exposed epitopes.

Polyclonal monospecific antibodies against Omp4

The Omp4 gene was amplified by PCR with primers that contained LIC-sites, and the PCR product was cloned into the pET-30 LIC vector (Novagen). The histidine tagged fusion protein was expressed by induction of the synthesis by IPTG and purified over a nickel column. The purified Omp4 protein was used for immunization of a rabbit (six times, 8 μg each time).

Use of rabbit polyclonal antibodies to recombinant Omp4 for detection of *Chlamydia pneumoniae* in paraffin embedded sections

The lungs of *C. pneumoniae* infected mice were obtained three days after intranasal infection. The tissue samples were fixed in 4% formaldehyde, paraffin embedded, sectioned and deparaffinized prior to staining. The sections were incubated with the rabbit serum diluted 1:200 in TBS (150 mM NaCl, 20mM Tris pH 7.5) for 30 min at room temperature. After wash two times in TBS the sections were incubated with the secondary antibody (biotinylated goat anti-rabbit antibodies) diluted 1:300 in TBS, followed by two times wash in TBS. The sections were stained with streptavidin-biotin complex (streptABComplex/AP, Dako) for 30 min washed and developed under microscopic inspection with chromagen + new fuchsin (Vector laboratories). The sections were counter stained with Hematoxylin and analyzed ny microscopy.

Immuno blotting analysis with hyperimmune monospecific rabbit anti-serum

The insert of pEX1-1 clone was amplified by PCR using primers containing LIC sites. The PCR product could therefore be inserted in the pET-32 LIC vector (Novagen, UK cat No. 69076-

1). Thereby the insert sequence of the pEX1-1 clone was
expressed in the new vector as a fusion protein, the part of
the fusion protein encoded by the pET-32 LIC vector had 6
histidine residues in a row. The expression of the fusion

5   protein was induced in this vector, and the fusion protein
could be purified under denaturing condition on a Ni2+ column
due to the high affinity of the histidine residues to
divalent cations. The purified protein was used for
immunization of a New Zealand white rabbit. After 6 times

10 intramuscular and 2 times intravenous immunization the serum
was obtained from the rabbit. Purified *C. pneumoniae* EB was
dissolved in SDS-sample buffer. Half of the sample was heated
to 100°C in the sample buffer, whereas the other half of the
sample was not heated. The samples were separated by

15 SDS-PAGE, and the proteins were transferred to
nitrocellulose, the serum was reacted with the strips. With
the samples heated to 100°C the serum recognized a high
molecular weight band of approximately 98 kDa. This is in
agreement with the predicted size of Omp5, of which the

20 pEX1-1 clone is a part, however, when the antibody was
reacted to the strip with unheated EB, the pattern was
different. Now a band was seen with a size of 75 kDa, in
addition weaker bands were observed above the band (Figure
10). These data demonstrate that Omp5 needs boiling in

25 SDS-sample buffer to be fully denatured and migrate with a
size as predicted from the gene product. When the samples
were not boiled, the protein was not fully denatured and less
SDS binds to the protein and it has a more globular structure
that will migrate faster in the acrylamide gel. The band

30 pattern looked identical to what was obtained with a
monoclonal antibody (MAb 26.1)(lane 6), we earlier have
described (Christiansen et al., 1994), reacting with the
surface of *C. pneumoniae* EB, but the antibody do not react
with the fully SDS denatured *C. pneumoniae* EB in

35 immunoblotting.

Experimental infection of C57 black mice

Due to the realization of the altered migration of the Omp4-7
proteins without boiling, we chose to analyse antibodies
against *C. pneumoniae* EBs after an experimental infection of
5   mice. To obtain antibodies from an infection caused by *C.
pneumoniae*, C57 black mice were inoculated intranasally with
10^7 CFI of *C. pneumoniae* under a light ether anaesthesia.
After 14 days of infection the serum samples were obtained
and the lungs were analysed for pathological changes. In two
10   of the mice a severe pneumonia was observed in the lung
sections, and in the third mouse only minor changes were
observed. The serum from the mice was diluted 1:100 and
reacted with purified EBs dissolved in sample buffer with and
without boiling. In the preparations that had been heated to
15   100°C the sera from two of the mice reacted strongly with
bands of 60/62 kDa and weaker bands of 55 kDa, but no
reaction was observed with proteins of the size of Omp4-7
(Figure 11). However, when the sera were reacted with the
preparation that had not been heated they all had a strong
20   reaction with a broad band of an approximate size of 75 kDa.
This is in agreement with the size of the Omp4-7 proteins in
the unheated preparation. Therefore, it could be concluded
that the epitopes of the Omp4-7 proteins recognized by the
antibodies after a *C. pneumoniae* infection were discontinuous
25   epitopes because the full denaturation of the antigen
completely destroyed the epitopes. The 75 kDa protein
observed in unheated samples is not Omp2 (Shown in
immunoblotting with an Omp2 specific antibody)

EXAMPLE 3

30   Comparison of Omp4-7 of *C. pneumoniae* with putative outer
membrane proteins (POMP) of *C. psittaci*

Longbottom et al. (1996) have published partial sequence from
98 to 90 kDa proteins from *C. psittaci*. They have entered the
full sequence of 5 genes in this family in the EMBL database.

They have named the genes "putative outer membrane proteins" (POMP) since their precise location was not determined. The family is composed of two genes that are completely identical, and two genes with high homology to these genes.
5   They calculated a molecular size of 90 and 91 kDa. The 5th encode a protein of 98 kDa. The sequence of the Omp4-7 proteins of *C. pneumoniae* were compared to the sequences of the *C. Psittaci* POMP proteins with the programme pileup in the GCG package. The amino acid homologies were in the range
10   of 51-63%. It is seen that the *C. pneumoniae* Omp4-5 proteins are most related to the 98 kDa POMP protein of *C. psittaci*. Interestingly, the 98 kDa *C. psittaci* POMP protein is more related to the *C. pneumoniae* genes than to the other *C. psittaci* genes. The repeated sequences of GGAI were conserved
15 . in the 98 kDa POMP protein, but only three GGAI repeats were present in the 90 and 91 kDa *C. psittaci* POMP proteins. For *C.psittaci* it has been shown that antibodies to these proteins seem to be protective for the infection.

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